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The arginine-vasotocin and serotonergic systems affect interspecific social behavior of client fish in marine cleaning mutualism

Zegni Triki[a*], Redouan Bshary[a], Alexandra S. Grutter[b] and Albert FH Ros[a]

[a] Behavioral Ecology Laboratory, Faculty of Science, University of Neuchâtel, Emile-Argand 11, 2000 Neuchâtel, Switzerland

[b] School of Biological Sciences, The University of Queensland, Brisbane QLD 4072, Australia

*Corresponding Author: Zegni Triki, email: zegni.triki@unine.ch

Abstract

Many species engage in mutualistic relationships with other species. The physiological mechanisms that affect the course of such social interactions are little understood. In the cleaning mutualism, cleaner fish *Labroides dimidiatus* do not always act cooperatively by eating ectoparasites, but sometimes cheat by taking bites of mucus from so-called "client" reef fish. The physiological mechanisms in these interspecific interactions, however, are little studied. Here, we focussed on three neuromodulator systems known to play important roles in intraspecific social behaviour of vertebrates to examine their role in clients' interspecific behaviour. We subjected the client fish *Scolopsis bilineatus* to ectoparasites and the exogenous manipulation of the vasotocin (AVT), isotocin (IT) and serotonin systems to test how this affects client willingness to seek cleaning and client aggression towards cleaners. We found that that a single dose of AVT agonist and a selective antagonist caused clients to seek proximity to cleaners, independently of ectoparasite infection. In contrast, in a direct encounter task, the selective blocker of serotonin $5HT_{2A/2C}$ receptors, Ketanserin (KET), made client reef fish more aggressive towards cleaners in the absence of cleaners' bites of mucus. IT did not yield any significant effects. Our results suggest that the AVT system plays a role in social affiliation towards an interspecific partner, while the serotonin system affects clients' acceptance of level of proximity to cleaner fish during interactions. These two systems, therefore, were apparently co-opted from intraspecific social interactions to affect the course of interspecific ones also.

INTRODUCTION

Social behaviour within species has long been a research focus in animal behaviour. A large group of chemical compounds that act centrally as neuromodulators act as proximate mechanisms by regulating social interactions either on short time scales (Hessler and Doupe, 1999; Hoare et al., 2004) or via life history effects (Ricklefs and Wikelski, 2002; Wingfield et al., 1998). Many of these compounds also act peripherally, regulating physiology and homeostatic processes (Adkins-Regan, 2005; Grinevich et al., 2016; Reeder and Kramer, 2005; Ricklefs and Wikelski, 2002; Wingfield et al., 1998). The function of many of these neuromodulators has apparently been conserved in evolution as they often yield similar responses within specific social contexts across species (see review O'Connell and Hofmann, 2011). Neuromodulators yield behavioural plasticity according to social context by affecting the output of neural circuits (Taborsky and Oliveira, 2012). Plasticity in output can be achieved in various ways, like changes in structural organisation (i.e. synaptic plasticity, receptors distribution and neural density), biochemical switching (i.e. interaction between neuromodulators) and social context feedback on endogenous levels of neuromodulators (see review by Oliveira, 2009).

The neuropeptides that have been studied extensively to explain the fine-tuned regulation of social behaviour are those classified as neurohypophysial peptides, especially the mammalian arginine vasopressin (AVP) and its non-mammalian homologue arginine vasotocin (AVT), and oxytocin (OT) and its non-mammalian homologue isotocin (IT). Both neuropeptide groups AVP/AVT and OT/IT can have peripheral hormonal effects and central neuromodulation effects (see review by Stoop,

2012). AVP/AVT has two types of receptors, the V1-type and the V2-type (V1a, V1b and V2 in mammals, Birnbaumer, 2000; V1a and V2 in fish, Kline et al., 2011; Konno et al., 2010; Lema et al., 2012). The system has multiple physiological roles. For example, AVP/AVT acts as a hormone in osmoregulation via vasoconstriction (Balment et al., 2006; Johnston et al., 1981; Schrier, 2006) and acts as a neuromodulator regulating intraspecific social behaviour. The expression of AVP receptors has been shown to vary with social context, both within species as well as within a population (Cushing et al., 2001; Phelps and Young, 2003). For example, AVT can modulate the aggressive behaviour in the bluehead wrasse especially via the AVT V1a receptors depending on the established territoriality of the treated individuals (Semsar et al., 2001). AVT may also affect behaviour via changes in concentration: in zebrafish, AVT has a U-shaped dose-dependent anxiolytic effect (Braida et al., 2012). Moreover, the neuronal densities are an important factor to consider since the behavioural outcome of AVT expression can differ in intensity regarding this factor. For example, aggressive behaviour in butterflyfish correlated positively with AVT neuron numbers (Dewan and Tricas, 2011).

OT/IT has strong structural and some functional similarities with AVP/AVT (Landgraf and Neumann, 2008). OT/IT plays primarily a physiological role as a hormone (OT in initiation of labour and lactation, Fuchs et al., 1982; Fuchs and Fuchs, 1984; Parker et al., 1991, and IT in osmoregulation and circadian cycles, Kleszczyńska et al., 2006; Gozdowska et al., 2006). However, from a behavioural perspective, the OT/IT neuromodulator underpins affiliative behavior depending on species, doses and social context. For example, in chimpanzee, endogenous OT increases after grooming but only with strongly bonded partners (Crockford et al., 2013). The behavioural ef-

fects of exogenous IT in fish are difficult to interpret: in goldfish, a single dose of centrally infused IT had partly opposing effects depending on whether subjects were more or less social to begin with (Thompson and Walton, 2004). In the cichlid *Neolamprologus pulcher,* IT injections affected territorial aggression as a positive function of opponent size and also caused more submissive behaviour (Reddon et al., 2012) while high and low dosages of IT (but not an intermediate dosage) caused reduced associations with conspecifics in a second study (Reddon et al., 2014). Also in *N. pulcher*, endogenous brain IT correlated positively with social affiliation and grouping behaviour (O'Connor et al., 2016).

In addition to the abovementioned neuromodulators, a well-studied system is the serotonergic pathway with its wide role in physiology (see review by Jonnakuty and Gragnoli, 2008) and in social behaviour regulation (Kiser et al., 2012; Montoya et al., 2012). Serotonin (5-HT) is a neurotransmitter that is mainly considered as an anti-depressant molecule in humans (see review Young, 2013). Many data show that serotonin is involved in regulating aggressive behaviour, regardless of species/taxa (humans, Kuepper et al., 2010; mice, Audero et al., 2013; birds, Dennis et al., 2013; reptiles, Summers et al., 2005 and fish, Weinberger II and Klaper, 2014).

Studies on the neuromodulation of social behaviour have tended to focus on social systems in an intraspecific context (Balment et al., 2006; Bernstein et al., 1983; Donaldson and Young, 2016). Whereas, interacting socially with heterospecifics might also be vital for some species. For example, many reef fish species improve their growth, body condition and population densities by interacting with marine cleaning organisms (Waldie et al 2010, Ros et al. 2011, Wagner et al. 2016). Howev-

er, how proximate physiological mechanisms might mediate social behaviour in interspecific social interactions is relatively less-studied.

An interesting animal social system is the well-studied mutualistic marine cleaning system of the cleaner wrasse *Labroides dimidiatus.* It consists of iterative interactions between a specialized wrasse and its partners, which include a highly diverse range of reef fishes. Fish clients frequently seek contact with *L. dimidiatus* to benefit from their cleaning service where cleaners feed on client ectoparasites and dead tissues off the client's skin (Grutter, 2000). Despite this, a conflict of interest can arise and threaten the stability of the relationship, as cleaner wrasse have a high preference for client's mucus (Grutter and Bshary, 2003). Cleaners may therefore cheat their clients by eating their mucus, an act that correlates with a client jolt immediately after a cleaner wrasse mouth contact (Bshary and Grutter 2002).

Several studies have assessed how specific neuromodulator systems affect cleaning behaviour in the cleaner wrasse, and hence levels of cooperation. Findings vary from inciting treated cleaners to engage more often in cleaning interactions (serotonin system, Paula et al., 2015; dopamine system, Messias et al., 2016) to suppressing the cleaners' willigness to clean (AVT system, Soares et al., 2012). However, no effects are detected in cleaners treated with the IT neuromodulator (Cardoso et al., 2015; Soares et al., 2012). The cleaners' willingness to cheat was only affected by manipulation of cortisol levels (Soares et al., 2014).

Clients cannot adjust their own levels of cooperation during interactions as their role is relatively passive, but they may affect the levels of cooperation expressed by cleaners. For example, clients decide to seek cleaners as a function of ectoparasite infection presence (Grutter 2001) and correlated physiological changes

(Triki et al., 2016), and they may respond to observations and/or experiences of cheating by cleaners with evasive actions like avoidance, flight and aggressive chasing (Bshary & Grutter 2002, Pinto et al. 2011). The role of neuromodulators in the clients' behaviour, however, remains unstudied, with the exception of Ros et al. (2012) who showed that blocking cortisol lead to reduced cleaner-visit frequencies. Whether other endocrine systems, known to influence intraspecific social behaviour in other organisms, may also affect client decisions in interactions with cleaners remains unexplored.

Here, we investigate how the exogenous manipulation of AVT, IT and serotonergic systems modulates clients' mutualistic behaviour. First, we hypothesised that AVT could affect client's interest to seek or invite for a cleaning interaction. Second, the IT system might have no role in interspecific social decisions as IT effects are so far only demonstrated in conspecifics (Reddon et al., 2014; Thompson and Walton, 2004). Finally, we chose the serotonergic system as a potential mediator of aggression in fish (Perreault et al., 2003; Weinberger II and Klaper, 2014). Serotonin has an inhibitory effect on impulsive aggression via the 5-HT$_2$ receptors (Coccaro and Kavoussi, 1997). And in order to estimate the well-documented client decisions to punish cleaners (Bshary et al. 2008), we hypothesised that 5-HT$_2$ receptors blockade would increase aggression.

To investigate the role of the aforementioned neuromodulator systems, we used the neuropeptide AVT and the selective blocker the AVT V1a receptor antagonist (Manning compound; see review by Manning et al., 2012), the neuropeptide IT, and ketanserin (KET) - a potent selective antagonist of serotonin receptors (Whitaker et al., 2011) involving the 5-HT2A/2C receptors (Leysen et al., 1982). The treated fish

were assigned to a laboratory experimental design that included two parts: (i) The first tested these neuromodulators, in interaction with exposure to parasites, using a choice task between two social stimuli (a cleaner wrasse and a non-cleaner wrasse). Parasite exposure was used because Grutter et al. (2001, 1999) showed that it is a key motivation for the client fish to seek an interaction with the cleaner wrasse. (ii) The second part consisted of direct cleaner-client interactions. We tested whether the neuromodulator systems of interest (AVT, IT, and serotonin) modulate a client's decision-making during encounters with cleaners, i.e. their willingness to invite for inspection, their willingness to allow interactions to continue, and their willingness to flee from them or aggressively chase and punish cleaners in response to cheating.

MATERIAL AND METHODS

FIELD SITE AND ANIMALS

The experiments were conducted at Lizard Island Research Station, Great Barrier Reef, Australia (14°40'08.0 S 145°27'34.0 E). During the period of August-September 2013, using barrier-nets and hand-nets, we caught 80 monocle bream *Scolopsis bilineatus* as client fish, 16 cleaner wrasse *Labroides dimidiatus* as the cleaner fish, and 16 pinstripe wrasse *Halichoeres melanurus* as the non-cleaner fish (the latter following Grutter 2001). These species were chosen because they were highly abundant on the reef. *H. melanurus* was chosen as a control stimulus for *L. dimidiatus* as this species is a non-cleaning wrasse species that has a similar size and shape to the cleaner wrasse.

Aquaria were mounted under shelters to protect fish from direct sunlight, were provided with continuously fresh seawater from the nearby reef, and were continuously aerated. In each aquarium, polyvinylchloride pipes (10 x 20 cm) were provided as shelter. *S. bilineatus* were housed individually in a blue opaque plastic bin (67 x 44 x 42 cm), which had a transparent window (20 x 20 cm) on the front to facilitate behavioural observations. Glass aquaria (67 x 37 x 38 cm) were used to house *L. dimidiatus* and *H. melanurus,* individually. *S. bilineatus* were daily fed with prawns pieces and both wrasses were fed with mashed prawns smeared on plastic plates (7 x 7 cm). Wrasse and *S. bilineatus* were acclimatized for 6 days prior to being tested.

NEUROMODULATORS' MANIPULATION

We had 5 treatments involving different compounds dissolved in saline: (a) the neuropeptide arginine vasotocin acetate-salt (AVT, V0130-Sigma), (b) isotocin (IT, H-2520-Bachem), (c) the V 1a receptors blocker the Manning compound (MC, V2255-Sigma), (d) the selective 5HT 2A/2C receptors antagonist ketanserin (+)-tartrate (KET, S006-Sigma), or (e) as a control, saline only. These compounds were injected intramuscularly, directly before each task. Each animal received in total three injections, from one of the 5 treatments, over the 5 days of experimentation. Two injections were linked to the first experiment that involved a client seeking the proximity of a cleaner when infected with parasites or uninfected, and the third injection was linked to the second experiment, which tested client behaviour during actual interactions with a cleaner. Dosages for AVT, IT and MC were 0.5 µg of the neuromodulator per gram of body weight (adapted from study by Santangelo and Bass, 2006). *S. bilineatus* weight was estimated from linear regression function from the body length (Nusbaumer et al., unpublished data). We used solutions of 1 mg of each compound dissolved in 5 ml of saline. For KET, the dosage was 10 µg per gram of body weight, using a solution of 20 mg in 95% saline and 0.5% ethanol (adapted from study by Whitaker et al., 2011).

EXPERIMENTAL SET-UP

Fish capture and experimentation was done in 4 separate periods, each for a period of 12 days: 6 days of acclimatization for all fishes, 5 days for the experiment, and at day 12 we tagged the fish by injecting subcutaneous visible implants (Northwest Marine Technology, INC) to avoid recapture of the same individuals, and we released

them to their respective site of capture. In each period, we tested 20 *S. bilineatus*, and had four *L. dimidiatus* and four *H. melanurus* as stimuli. Directly after capture, fish were deparasitized using a freshwater bath for three minutes (Jones and Grutter, 2005) followed with an anti-helminthic bath of Praziquantel overnight with aeration (ICN Biomedicals Inc., Aurora, OH, USA) (1:100,000).

Clients' preference test

This test was adapted from a design by Grutter (2001). Similar to her design, two large oval tanks (215 x 100 x 50 cm) were used: one with cultured gnathiid isopod ectoparasites and the other free of gnathiids at Lizard Island Research Station (Grutter, 2001). Gnathiids swim freely in the tank culture in search of hosts, whom they attack and feed on their blood (Grutter 2003). The species *S. bilineatus* is a relatively common host of gnathiid isopods at Lizard Island (Grutter, 1994). In each long side of the tank, a transparent aquarium (67 x 37 x 38 cm) was mounted. During trials, one *L. dimidiatus* and one *H. melanurus* were already held in each of the test aquaria inside the tank before introducing the focal individual in the tank (Fig. 1). Prior to each trial, the focal individual received an injection with the test compound and was placed in the middle of one of the test tanks where both social stimuli the cleaner and the non-cleaner wrasses were visible but not accessible through the glass aquaria inside the tank. The focal fish behaviour was recorded for 30 min with a GoPro® Hero2 camera mounted above the tank. We chose a 30 min test duration since we were testing fast-acting neuromodulators. The order of treatments and the placement of stimuli fish were counter-balanced. After each trial, fish from the parasite-exposure treatment were captured and kept in a bucket with seawater and air-

flow for 30 min. This allowed attached gnathiids to finish their blood meal and to drop off. As a last step before returning fish back to their aquaria we put them briefly (1 min) in a bucket with freshwater to remove any potentially remaining attached gnathiids. Control fish were treated in a similar way. Finally, we counted detached gnathiids and returned them to the parasite culture tank. No gnathiids were found on the control fish. The parasite load was estimated as the density of parasites per body surface area. Body surface area was estimated using ln(area)= 2.19 + 0.67 ln(weight) for *S. bilineatus* (Grutter, 1994). Each focal individual was tested in both test tanks with a 4 days interval period between the two tests.

Cleaner-client interaction test

To test the effects of neuromodulator treatment on direct interactions between clients and cleaners, the *L. dimidiatus* individual was released in the holding bin of its respective client *S. bilineatus* from the previous test. Before releasing the cleaner, the *S. bilineatus* received the same treatment injection it had received when it was tested for its interspecific social preferences. Behaviour was recorded for 15 min using a cam-recorder Handycam® Sony HDD placed in front of window of the bin.

BEHAVIOURAL ANALYSIS

Behaviour was analysed from videos using the software CowLog (Hänninen and Pastell, 2009). In the "clients' preference test" we quantified the time spent by the focal fish in the designated zones around each of the two stimulus fish. For this purpose,

the tank was divided in three zones: the side around *L. dimidiatus*, the side around *H. melanurus*, or the middle of the tank (Fig. 1). We measured client's willingness to be with the cleaner wrasse by calculating the time spent on the cleaner wrasse side divided by the total time spent on either the cleaner wrasse side and the non-cleaner wrasse side (a proportion). The resulting index could thus vary from zero to 1, with 0.5 defined as a random choice.

In the "cleaner-client interaction test", we analysed the behavioural interactions between the two fish. Here we calculated: (i) total duration of the cleaner's interactions; (ii) quality of the cleaner's interactions: duration of the tactile stimulations provided by *L. dimidiatus* per total cleaner interaction duration; number of client jolts caused by cleaner fish mouth contact; (iii) aggressive punishment of the cleaner: frequency per interaction duration of chasing behaviour following clients' jolts (Bshary and Grutter, 2002); (iv) unprovoked aggression: chasing behaviour that occurred in the absence of previous client jolting; and (v) spontaneous aggression: chasing behaviour that occurred outside the cleaner-client cleaning interaction.

DATA ANALYSIS

Statistical analyses were conducted with the software RStudio© (version 3.0.2. 2013-09-25). We employed parametric tests in instances where the assumptions of a normal distribution and homogeneity of variance were met. We used linear mixed effect models (LME) from the package (nlme). In the models, the identity of the clients and the cleaners were classified as random factors in the client's preference test and the client-cleaner interaction test, respectively. For the client's preference task and the response "Client willingness to spend time next to a cleaner wrasse",

we tested for the effects of neuromodulator treatment, parasite treatment, and the interaction between the two. If non-significant, the interaction term was then dropped from the model, and the model re-analysed for contrasts by calling the `summary ()` function.

For the client-cleaner interaction task, we tested for the impact of neuro-modulators on the different cleaning behaviours (cleaning duration, the proportion of the provided tactile stimulations, and the different aggressive behaviours). Proportion data from the client's preference test (proportion of time spent next cleaner wrasse) and from the client-cleaner interaction test (client-cleaner interaction duration and duration of receiving tactile stimulations from the cleaner wrasse) were transformed using the arcsine square root. The skewed frequency of unprovoked aggression data from the client-cleaner interaction test was power transformed. For data where the assumptions for the parametric tests were not met (punishment and spontaneous aggression), we opted for the nonparametric test of one-way Kruskal-Wallis analysis of variance from the Agricolae Package (R language, version 1.1-8).

Results

Clients' preference test

There was no significant interaction between the neuromodulator and parasite treatments on the willingness of *S. bilineatus* to be on the side next to the cleaner (Two-way ANOVA: X^2 (4,71) = 1.68, *p*=0.792). After excluding the interaction term from the model, the effect of parasite treatment was not significant (LME: t(75) = -0.52, *p*=0.599). In contrast, the effect of the neuromodulator treatment AVT was significant (LME: t(74) = 2.47, *p*=0.015; Fig. 2) with AVT appearing to cause a signifi-

cant increase in client willingness to seek a cleaner. The other compounds showed no significant effect, though there was a nearly significant tendency that MC might also increase the clients' willingness to seek cleaners (LME: IT: t(74) = 0.70, p = 0.485; MC: t(74) = 1.98, p=0.051; KET: t(74) = 1.46, p=0.148; Fig. 2).

Client-cleaner interaction

On average, a large proportion of the cleaning service offered by the cleaner wrasse when in direct contact with the clients involved tactile stimulation (interaction duration: N=72 clients; mean ± SE 382.85 ± 33.67 seconds; percentage of time spent providing tactile stimulation per interaction: mean ± SE; 32.40 ± 3.73 %). The frequency of mouth contacts was relatively low (mean ± SE; 2.37 ± 0.28 mouth contact per 100 seconds of interaction, N=72), and consequently the frequency of jolts was low as well (mean ± SE; 0.27 ± 0.07 mouth contact followed by jolt per 100 seconds of interaction). No fleeing behaviour in response to cleaners' cheating, nor client fish posing, was observed.

The client-cleaner interaction duration (LME: IT: t(52)= -0.56, p=0.575; KET: t(52)=0.44, p=0.655; MC: t(52)= -0.06, p=0.950; AVT: t(52)=0.36, p=0.717; Fig. 3a) and the proportion of time providing tactile stimulation (LME: IT: t(52)= -0.48, p=0.627; KET: t(52)=1.29, p=0.201; MC: t(52)= -0.32, p=0.746; AVT: t(52)=0.78, p=0.436; Fig. 3b) were unaffected by the neuromodulator treatment. Neuromodulator treatment did not affect spontaneous aggression that occurred outside the cleaner-client interaction (One-way Kruskal-Wallis test: x^2 (4)= 4.09, IT vs. control: p=0.23; KET vs. control: p=0.80; MC vs. control: p=0.74; AVT vs. control: p=0.23; Fig. 4a). However, unprovoked aggression was affected by the neuromodulator treat-

ment, with the group of fish treated with KET having significant increased levels of unprovoked aggression (LME: KET: t(52)=2.55, *p*=0.0137), while the other treatments had no significant effect (IT: t(52)=0.05, *p*=0.9559; MC: t(52)=0.71, *p*=0.4782; AVT: t(52)=-1.04, *p*=0.3021; Fig. 4b). We found no significant changes in punishment behaviour of clients as a function of neuromodulator treatment (One-way Kruskal-Wallis test: x^2 (4)= 2.14, IT vs. control: *p*=0.84; KET vs. control: *p*=0.89; MC vs. control: *p*=0.89; AVT vs. control: *p*=0.28; Fig. 4c).

DISCUSSION

This study aimed to explore how a set of neuromodulator pathways underpins interspecific social behaviour of a client reef fish in a marine cleaning mutualism system. Specifically, we tested the involvement of these neuromodulators in clients' decision-making to seek cleaner wrasse proximity with and without parasite infection, their willingness to invite a cleaner for cleaning interaction, their willingness to interact longer with a cleaner and their expression of potential aggression by punishing biting cleaners in direct encounters.

Clients' preference to seek a cleaner proximity

Our findings show that the single dose of the neuromodulator AVT positively influenced the willingness of clients to be near a cleaner wrasse. Surprisingly, its selective blocker, the AVT V1a receptors blocker MC, instead of having an opposite outcome to the AVT agonist, yielded a similar effect. This is not the first time that AVT and MC have yielded an effect in the same direction: in zebrafish the two compounds decreased individuals' sociality (Lindeyer et al., 2015), while in the cleaner wrasse they decreased cleaning inspection durations of their clients (Soares et al., 2012). One po-

tential explanation that needs further investigation is that the V2 receptors might play a key role for the expression of clients seeking cleaners: in that case, blocking the V1A receptor with the antagonist could have caused a similar increase in AVT binding to V2 as did the injection of AVT. However, as clients' AVT basal levels were unknown, and since AVT has dose-dependent effects (Santangelo and Bass, 2006), our findings following exogenous manipulation with single doses of AVT and MC are currently preliminary. Alternatively, exogenous and endogenous AVT might have opposite effects and therefore administering exogenous AVT or blocking endogenous AVT may have yielded similar effects. Clearly, the association between AVT and social behaviour is rather complex as AVT is not restricted to a singular role but rather acts as a multitask neuromodulator that is strongly fine-tuned by the social environment (Goodson and Bass, 2001; Greenwood et al., 2008; Santangelo and Bass, 2006; Semsar et al., 2001; Soares et al., 2012). Indeed, in the cleaning mutualism system the same AVT and MC injections in cleaners can facilitate social approach in an intraspecific context towards partners, but inhibit interaction initiations of cleaners with clients in an inter-specific context (Soares et al., 2012). AVT is known as well for its role in affecting social behaviour in other contexts than social affiliation. For instance, respectively AVT and MC increased and/or decreased aggression in damselfish (Santangelo and Bass, 2006) and territoriality as a function of the social status in the blueheaded wrasse males (Semsar et al., 2001), while brain AVT expression in males of a territorial African cichlid was higher in the posterior preoptic area than in the anterior preoptic area, and the opposite was true for non-territorial males (Greenwood et al., 2008). Thus, the emerging picture is that AVT has diverse effects depending on species and social context. The main conclusion from our study is that

manipulation of the AVT system by giving an agonist or an antagonist affects the client decision-making process regarding the approach of cleaners, which shows that the AVT system is involved in the regulation of interspecific interactions. AVT and its V1A receptor blocker made clients seek cleaners over non-cleaners.

There was no significant effect of the IT system on the client's preference to be near a cleaner wrasse. In addition to the similar findings of Cardoso and colleagues (2015), our study suggests that the IT system does not play a role in the regulation of a marine cleaning mutualism. However, other research conducted on IT/OT systems confirms their involvement in intraspecific social behaviour (see reviews by Campbell, 2008; Lukas et al., 2011; Reddon et al., 2012; Thompson and Walton, 2004). Therefore, the IT system may mediate social behaviour exclusively in individuals with strong established social bonds and hence be tightly linked to an intraspecific context (i.e., in monogamous prairie voles: Insel and Hulihan, 1995; Burkett et al., 2016; and in squirrel monkeys: Winslow and Insel, 1991), except for peculiar interspecific contexts like between dogs and humans (Odendaal and Meintjes, 2003). The individuals tested in the present study are most likely strangers to each other, which excludes any prior established social bonding. An alternative for future research would be to study the IT system in familiar individuals such as a cleaner wrasse with its resident clients, as it has been shown that relationships have to be established first for normal cleaning interactions to occur (Bshary, 2002).

The selective blocker of the serotonin receptors KET showed a non-significant effect in influencing clients to seek cleaners. Seeking of cleaners may be expected if the serotonergic function in intraspecific social behaviour has indeed been co-opted for interspecific social interactions, as suggested by our results on client aggression

towards cleaners (see discussion below). As with IT, it would be interesting for future studies to evaluate the potential role of serotonin in clients that are exposed to cleaners with whom they have established relationships.

Ectoparasites had no significant effect on client choices. Overall, clients did not prefer to seek cleaners over non-cleaners in the parasite infection treatment, independently of the administration of neuromodulators. This is in contrast to an earlier study in which the client reef fish *Hemigymnus melapterus,* in a similar design, displayed high preference towards cleaners over non-cleaners in the parasite treatment (Grutter, 2001). In that study, a prior acclimatization period in the parasite and control tanks occurred (1 day), whereas in the present study there was no acclimatization period. Furthermore, *H. melapterus* were held in captivity in for a 5 month period, whereas, here, *S. bilineatus* were held for only 6 to 10 days in captivity before testing. The differences in results could thus be due to the use of different client species but alternatively due to our fish being more stressed by the procedure (Grutter and Pankhurst, 2000).

Clients' direct interactions with cleaners

None of the neuromodulators affected the time clients spent interacting with cleaner wrasse. However, these results do not necessarily contradict the results from our first experiment as cleaners rather than clients may be the main decision-makers about interactions in a confined aquarium. More importantly, the manipulation of the serotonergic system with KET affected clients' unprovoked aggression, i.e. aggression towards cleaners that were either approaching or behaving cooperatively. In the latter case, cleaners were often attacked aggressively by clients while cleaners provided them with tactile stimulation using their pelvic fins, a behaviour that is

normally beneficial to clients as it reduces stress (Soares et al., 2011). Our outcomes fit previous findings on serotonin role in mediating aggressive behaviour in fish, bluehead wrasse receiving an agonist of serotonin exhibited lower levels of aggression towards conspecific intruders (Perreault et al., 2003). In contrast to unprovoked aggression, spontaneous aggression and aggression in response to own jolts, cheating by cleaners was unaffected by neuromodulator treatment. The latter result is not conclusive, however, as the low number of total jolts prevented the possibility for significant results. Thus, our study did not allow us to evaluate whether any of the substances tested modulates client decisions on punishing cheating cleaners.

Not much is known about the serotonergic system in fish, especially regarding receptors and their distribution. In several vertebrate species including humans, many serotonin receptors are indeed involved in modulating aggressive behaviour (Higley et al., 1996; Kuepper et al., 2010; Liechti et al., 2000). Acting selectively on specific receptors may increase or decrease aggression depending on the administered molecule and the social cue (Summers et al., 2005). Our findings suggest that the neurotransmitter serotonin system mediates client's tolerance to close contact with cleaners. However, it remains unclear how exactly the serotonin system is activated before and/or during the cleaning interactions.

The other neuromodulators tested did not affect the course of interactions between cleaners and clients. This contrasts with previous manipulations of cleaner fish physiology, in which the AVT system had a major effect on the cooperative quality of the cleaners (Cardoso et al., 2015; Soares et al., 2012). However, the behavioural role of AVT could rely on the phylogeny of the system and on the context. For instance, in the cleaner wrasse brain, more precisely in the gigantocellular preoptic

area, the AVT neurons are rare and less dense compared to a non-cleaner wrasse (Mendonça et al., 2013). Also, injecting neuropeptide AVT into different fish species and assigning them to the same behavioural task can yield different behavioural responses (Perrone et al., 2010). Thus, the comparison of neuromodulator manipulation outcomes from different species and taxa should be conservative (see review by Goodson and Bass, 2001).

Conclusions

Our findings provide new insights into how different neuromodulator pathways underpin social behaviour in interspecific social interactions. Even after a relatively short period of acclimatization, the AVT system mediates clients' willingness to seek a cleaner wrasse. Our results fit previous evidence that the AVT circuits may modulate the behavioural output in non-linear ways (Braida et al., 2012; see review Godwin and Thompson, 2012; Santangelo and Bass, 2006), which makes predicting the direction of effects very difficult. As a consequence, in future studies it will be helpful to quantify the endogenous baseline levels of AVT to better interpret the effects of manipulations. Our other major result was that the serotonergic system appears to affect the course of interactions by regulating client tolerance to proximity to cleaners. In conclusion, it appears that systems that have evolved to regulate intraspecific social behaviour have been co-opted to also regulate interspecific social behaviour, though precise predictions about effects remain unresolved at this point.

ETHICAL NOTE

The University of Queensland Animal Ethics Committee (AEC) approved the study.

All animals were released at their site of capture.

Conflict of Interest

All authors declare that they have no conflict of interest.

Figures captions:

Figure 1. The experimental setup for the clients' preference test. Experimental tanks (234 x 124 x 53 cm) contain: two social stimuli: a cleaner wrasse and a non-cleaner wrasse, held in separate glass aquaria (64 x 30 x 40 cm) at opposite ends of the tank and two boxes in the middle (58 x 38 x 33 cm) filled with dead coral rubble used as shelter for gnathiids in the parasite tank and left empty in the non-parasite tank. The choice behaviour of the treated client reef fish was recorded for 30 minutes with a camera attached 65 cm above the tank surface. Dashed lines show the three areas used in the analyses of client fish choice.

Figure 2. Clients' preference to seek a cleaner proximity. Boxplots of median, inter-quartile and ranges of: percentage of time recorded for client fish in the cleaner wrasse's side per rate of time spent on either cleaner side or non-cleaner side in the

choice tank. Dashed line shows the prediction of the null hypothesis that clients choose randomly in the control group and it is represented as 50% of time in each wrasse side. Neuromodulator treatment abbreviations are: IT, isotocin; KET, ketanserin; MC, manning compound; AVT, arginine vasotocin. *: p <0.05.

Figure 3. Clients' direct interactions with cleaners. Boxplots of median, interquartile and ranges of: (**a**) the interaction duration in seconds and (**b**) the proportion of the time spent giving tactile stimulation during these interactions in the five groups of treatments. Neuromodulator treatment abbreviations are: IT, isotocin; KET, ketanserin; MC, manning compound; AVT, arginine vasotocin. n.s.: non-significant differnces.

Figure 4. Clients' aggressive behaviour towards cleaners in direct interactions. Boxplots of median, interquartile and ranges of (**a**) spontaneous aggression, the frequency of attacks occurring in 100s time duration of no-interaction, (**b**) unprovoked aggression, in 100s time duration of interaction and (**c**) provoked aggression, the rate of clients attacking cleaners directly after mouth contact estimated as the percentage of attacks per rate of client's jolts; red dots show the data points. Neuromodulator treatment abbreviations are: IT, isotocin; KET, ketanserin; MC, manning compound; AVT, arginine vasotocin. *: p <0.05. n.s.: non-significant differnces.

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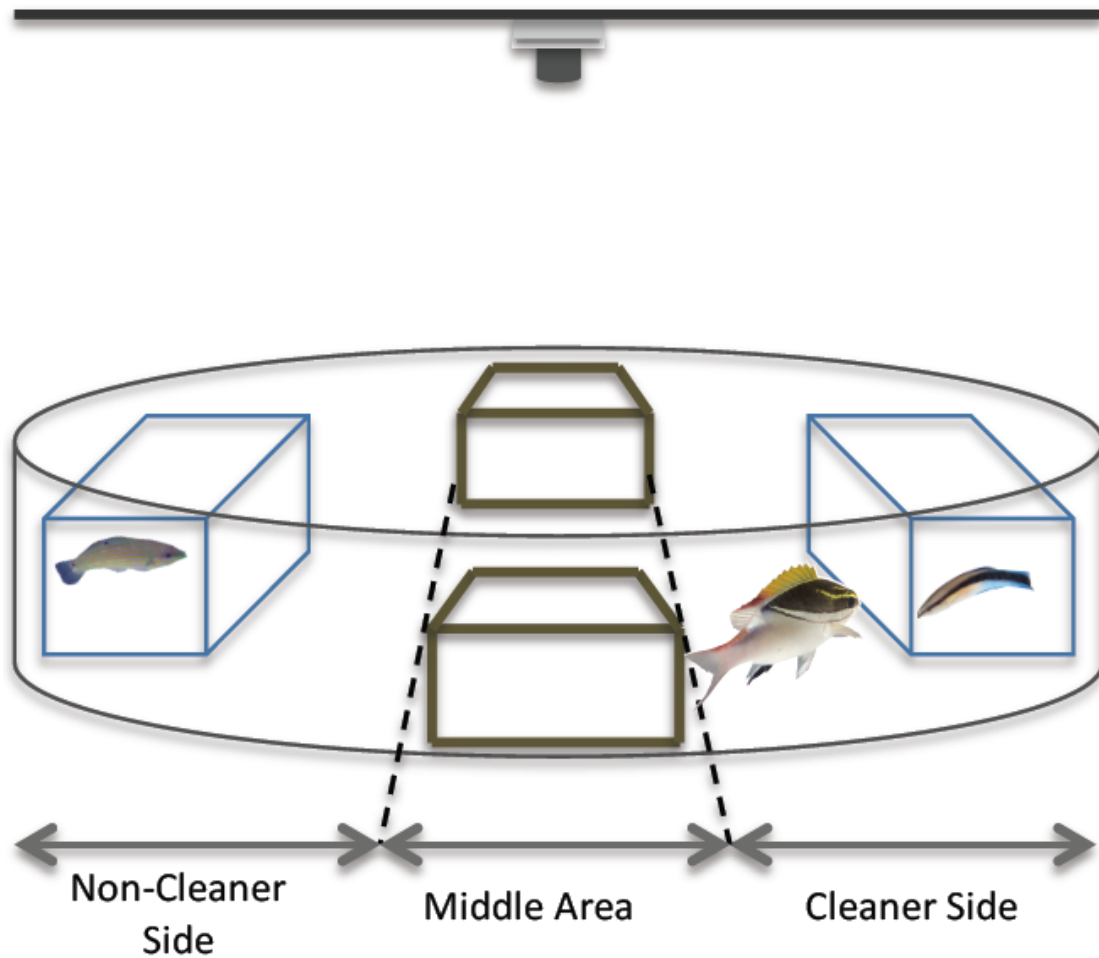
Figure 1

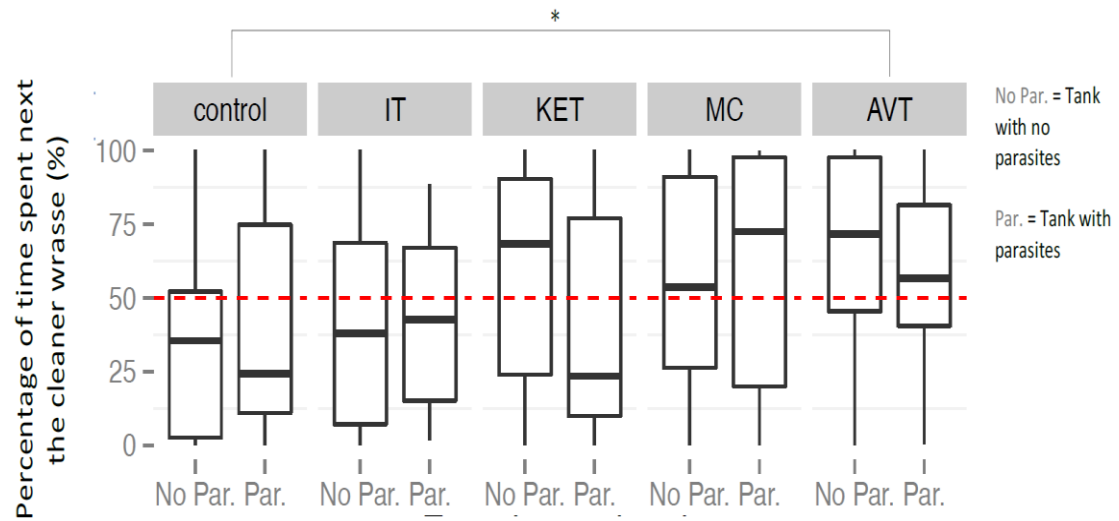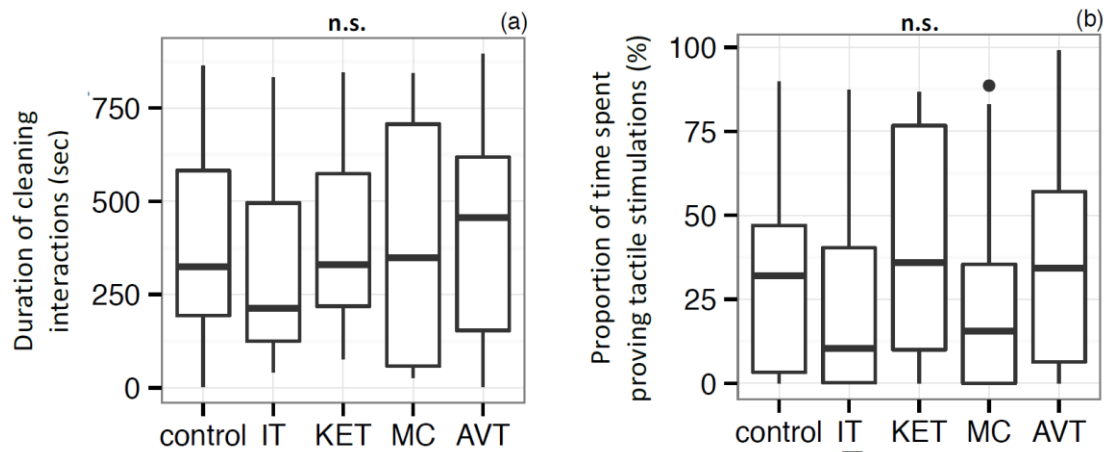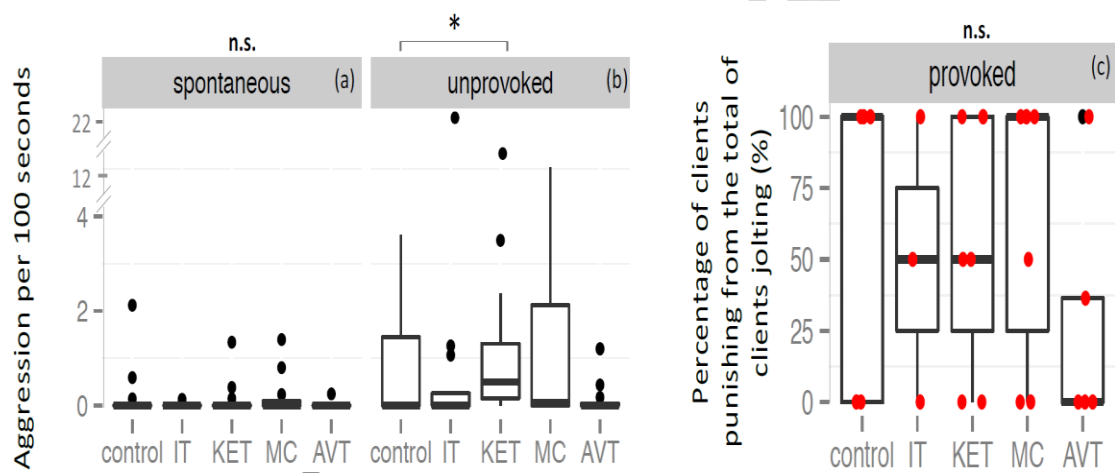Non-Cleaner Side   Middle Area   Cleaner Side

Figure 2

Figure 3

Figure 4

Highlights

- In the marine cleaning mutualism, we exogenously manipulated three neuromodulator systems to study client fish interspecific behaviour

- Agonist and antagonist of vasotocin enhance clients' willingness to choose cleaner wrasse over a non-cleaner wrasse

- The serotonin 5HT 2A/2C receptors antagonist ketanserin KET increased clients' intolerance to cleaners proximity

- The isotocin system had no role suggesting its exclusive role in an intraspecific context